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(54) Title: ACYLATED INSULIN (57) Abstract <p>The present invention relates to protracted human insulin derivatives in which the A21 and the B3 amino acid residues are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the B30 amino acid residue is a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ε-amino group of Lys^{B29}; or b) the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in any of which cases the ε-amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is present, then the insulin derivative is always present as a Zn²⁺ complex.</p>		

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ACYLATED INSULIN

FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be

immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., Studies on the immunogenecity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection, and thus the timing of the medication, depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ϵ -amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, US Patent No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the

insulin molecule has a carboxyaroyl group. No specifically N^{εB29}-substituted insulins are disclosed.

According to GB Patent No. 1.492.997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at N^{εB29} has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ε-amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position have an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulfide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulfide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain human insulin derivatives, wherein the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

5 Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ -amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent; and any Zn²⁺ complexes thereof, provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn²⁺ complex.

20 In one preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is not deleted, then 2-4 Zn²⁺ ions are bound to each hexamer of the insulin derivative.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3

amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; Phe^{B1} may be deleted; and the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn²⁺ ions are bound to each insulin hexamer.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic amino acid having at least 10 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a straight chain, saturated, aliphatic α -amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is D- or L-N^ε-dodecanoyllysine.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino decanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino undecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino dodecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tridecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino tetradecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino pentadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α -amino hexadecanoic acid.

In another preferred embodiment, the invention relates to a
5 human insulin derivative in which the B30 amino acid is an α -amino acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is
15 Gly.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment, the invention relates to a
20 human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

25 In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

5 In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group, branched or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a
15 human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a
20 human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment, the invention relates to a
25 human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 10 to 16 carbon atoms.

In another preferred embodiment, the invention relates to a
30 human insulin derivative in which the ϵ -amino group of Lys⁸²⁹ has

a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oxyethylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ -amino group of Lys^{B29} has
5 a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 2 Zn²⁺ ions.

10 In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 3 Zn²⁺ ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 4
15 Zn²⁺ ions.

In another preferred embodiment, the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a
20 pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

25 In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an

insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no Zn^{2+} ions are bound are the following:

- N^{εB29}-tridecanoyl des(B30) human insulin,
- 5 N^{εB29}-tetradecanoyl des(B30) human insulin,
- N^{εB29}-decanoyl des(B30) human insulin,
- N^{εB29}-dodecanoyl des(B30) human insulin,
- N^{εB29}-tridecanoyl Gly^{A21} des(B30) human insulin,
- N^{εB29}-tetradecanoyl Gly^{A21} des(B30) human insulin,
- 10 N^{εB29}-decanoyl Gly^{A21} des(B30) human insulin,
- N^{εB29}-dodecanoyl Gly^{A21} des(B30) human insulin,
- N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- 15 N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-tridecanoyl Ala^{A21} des(B30) human insulin,
- N^{εB29}-tetradecanoyl Ala^{A21} des(B30) human insulin,
- N^{εB29}-decanoyl Ala^{A21} des(B30) human insulin,
- N^{εB29}-dodecanoyl Ala^{A21} des(B30) human insulin,
- 20 N^{εB29}-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
- N^{εB29}-tridecanoyl Gln^{B3} des(B30) human insulin,
- 25 N^{εB29}-tetradecanoyl Gln^{B3} des(B30) human insulin,
- N^{εB29}-decanoyl Gln^{B3} des(B30) human insulin,
- N^{εB29}-dodecanoyl Gln^{B3} des(B30) human insulin,
- N^{εB29}-tridecanoyl Gly^{A21} human insulin,
- N^{εB29}-tetradecanoyl Gly^{A21} human insulin,
- 30 N^{εB29}-decanoyl Gly^{A21} human insulin,
- N^{εB29}-dodecanoyl Gly^{A21} human insulin,
- N^{εB29}-tridecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^{εB29}-tetradecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^{εB29}-decanoyl Gly^{A21} Gln^{B3} human insulin,
- 35 N^{εB29}-dodecanoyl Gly^{A21} Gln^{B3} human insulin,
- N^{εB29}-tridecanoyl Ala^{A21} human insulin,

- $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Gln^{B3} human insulin,
5 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gln^{B3} human insulin,
10 $N^{\epsilon B29}$ -decanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gln^{B3} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Glu^{B30} human insulin,
15 $N^{\epsilon B29}$ -dodecanoyl Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} Glu^{B30} human insulin,
20 $N^{\epsilon B29}$ -tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Glu^{B30} human insulin,
25 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
30 $N^{\epsilon B29}$ -decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tridecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -tetradecanoyl Gln^{B3} Glu^{B30} human insulin,
 $N^{\epsilon B29}$ -decanoyl Gln^{B3} Glu^{B30} human insulin and
35 $N^{\epsilon B29}$ -dodecanoyl Gln^{B3} Glu^{B30} human insulin.

Examples of preferred human insulin derivatives according to the present invention in which two Zn^{2+} ions are bound per insulin hexamer are the following:

- (N^εB29-tridecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- 5 (N^εB29-tetradecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- 10 (N^εB29-decanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- 15 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 2Zn²⁺,
- 20 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- 25 (N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl human insulin)₆, 2Zn²⁺,
- 30 (N^εB29-decanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-dodecanoyl human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-tetradecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-decanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- 35 (N^εB29-dodecanoyl Gly^{A21} human insulin)₆, 2Zn²⁺,
- (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,

- (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 5 (N^εB29-tetradecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 10 (N^εB29-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 15 (N^εB29-dodecanoyl Gln^{B3} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Glu^{B30} human insulin)₆, 2Zn²⁺,
 20 (N^εB29-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 25 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 30 (N^εB29-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 35 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺,

(N^εB²⁹-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺ and
 (N^εB²⁹-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 2Zn²⁺.

Examples of preferred human insulin derivatives according to the present invention in which three Zn²⁺ ions are bound per insulin hexamer are the following:

- (N^εB²⁹-tridecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl des(B30) human insulin)₆, 3Zn²⁺,
- 10 (N^εB²⁹-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 15 (N^εB²⁹-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- 20 (N^εB²⁹-decanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 25 (N^εB²⁹-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 3Zn²⁺,
- 30 (N^εB²⁹-tridecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tetradecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-decanoyl human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-dodecanoyl human insulin)₆, 3Zn²⁺,
- (N^εB²⁹-tridecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
- 35 (N^εB²⁹-tetradecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,

- (N^εB29-decanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 5 (N^εB29-decanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 10 (N^εB29-dodecanoyl Ala^{A21} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} human insulin)₆, 3Zn²⁺,
 15 (N^εB29-tridecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gln^{B3} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 20 (N^εB29-tetradecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 25 (N^εB29-decanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 30 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Glu^{B30} human insulin)₆, 3Zn²⁺,
 35 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,

- (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺ and
 5 (N^εB29-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 3Zn²⁺.

Examples of preferred human insulin derivatives according to the present invention in which four Zn²⁺ ions are bound per insulin hexamer are the following:

- (N^εB29-tridecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 10 (N^εB29-tetradecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 15 (N^εB29-decanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gly^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 20 (N^εB29-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} des(B30) human insulin)₆, 4Zn²⁺,
 25 (N^εB29-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 30 (N^εB29-tetradecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-decanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-dodecanoyl Gln^{B3} des(B30) human insulin)₆, 4Zn²⁺,
 (N^εB29-tridecanoyl human insulin)₆, 4Zn²⁺,
 (N^εB29-tetradecanoyl human insulin)₆, 4Zn²⁺,
 35 (N^εB29-decanoyl human insulin)₆, 4Zn²⁺,

- $(N^{\epsilon B29}\text{-dodecanoyl human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Gly}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Gly}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Gly}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
5 $(N^{\epsilon B29}\text{-dodecanoyl Gly}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
10 $(N^{\epsilon B29}\text{-tridecanoyl Ala}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Ala}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Ala}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Ala}^{A21}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Ala}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
15 $(N^{\epsilon B29}\text{-tetradecanoyl Ala}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Ala}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Ala}^{A21}\text{ Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
20 $(N^{\epsilon B29}\text{-decanoyl Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Gln}^{B3}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
25 $(N^{\epsilon B29}\text{-dodecanoyl Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Gly}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Gly}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Gly}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Gly}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
30 $(N^{\epsilon B29}\text{-tridecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tetradecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Gly}^{A21}\text{ Gln}^{B3}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-tridecanoyl Ala}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
35 $(N^{\epsilon B29}\text{-tetradecanoyl Ala}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-decanoyl Ala}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$
 $(N^{\epsilon B29}\text{-dodecanoyl Ala}^{A21}\text{ Glu}^{B30}\text{ human insulin})_6, 4Zn^{2+},$

- (N^εB²⁹-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB²⁹-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB²⁹-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB²⁹-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 5 (N^εB²⁹-tridecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB²⁹-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺,
 (N^εB²⁹-decanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺ and
 (N^εB²⁹-dodecanoyl Gln^{B3} Glu^{B30} human insulin)₆, 4Zn²⁺.

BRIEF DESCRIPTION OF THE DRAWINGS

- 10 The present invention is further illustrated with reference to the appended drawings wherein

Fig. 1 shows the construction of the plasmid pEA5.3.2;

Fig. 2 shows the construction of the plasmid pEA108; and

Fig. 3 shows the construction of the plasmid pEA113.

15 DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p. 3558 (1968).

- 20 In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used:

- DMSO for dimethyl sulphoxide, DMF for dimethylformamide, Boc for tert-butoxycarbonyl, RP-HPLC for reversed phase high
 25 performance liquid chromatography, X-OSu is an N-hydroxysuccinimid ester, X is an acyl group, and TFA for trifluoroacetic acid.

Preparation of lipophilic insulin derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin derivatives featuring in position B30 an amino acid residue which can be coded for by the genetic code, e.g. threonine (human insulin) or alanine (porcine insulin).

1.1 Starting from human insulin.

Human insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc human insulin, i.e., human insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ -amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, N ^{ϵ B29}-X human insulin, is isolated.

1.2 Starting from a single chain insulin precursor.

A single chain insulin precursor, extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and in which the bridge from B30 to A1 is an arginine residue, i.e. a compound of the general formula Ext-Arg-B(1-30)-Arg-A(1-21), can be used as starting material. Acylation of this starting material with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ -amino group of Lys^{B29} and in the N-terminal amino group of the precursor. On treating this acylated precursor of the formula (N ^{ϵ B29}-X),X-Ext-Arg-B(1-30)-

Arg-A(1-21) with trypsin in a mixture of water and a suitable water-miscible organic solvent, e.g. DMF, DMSO or a lower alcohol, an intermediate of the formula $(N^{\epsilon B29}-X), Arg^{B31}$ insulin is obtained. Treating this intermediate with carboxypeptidase 5 B yields the desired product, $(N^{\epsilon B29}-X)$ insulin.

2. Insulin derivatives with no amino acid residue in position B30, i.e. des(B30) insulins.

2.1 Starting from human insulin or porcine insulin.

On treatment with carboxypeptidase A in ammonium buffer, human 10 insulin and porcine insulin both yield des(B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc des(B30) insulin, i.e., des(B30) insulin in which the N-terminal end of both chains are protected by a Boc-group. After 15 an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ -amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, 20 $(N^{\epsilon B29}-X)$ des(B30) insulin, is isolated.

2.2 Starting from a single chain human insulin precursor.

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and which has a bridge from B30 to A1 25 can be a useful starting material. Preferably, the bridge is a peptide of the formula Y_n -Arg, where Y is a codable amino acid except lysine and arginine, and n is zero or an integer between 1 and 35. When $n > 1$, the Y's may designate different amino acids. Preferred examples of the bridge from B30 to A1 are: 30 AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No.

163529). Treatment of such a precursor of the general formula Ext-Arg-B(1-30)-Y_n-Arg-A(1-21) with a lysyl endopeptidase, e.g. Achromobacter lyticus protease, yields Ext-Arg-B(1-29) Thr-Y_n-Arg-A(1-21) des(B30) insulin. Acylation of this intermediate
 5 with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ε-amino group of Lys^{B29}, and in the N-terminal amino group of the A-chain and the B-chain to give (N^{εB29}-X) X-Ext-Arg-B(1-29) X-Thr-Y_n-Arg-A(1-21) des(B30) insulin. This intermediate on
 10 treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, (N^{εB29}-X) des(B30) human insulin.

Data on N^{εB29} modified insulins.

Certain experimental data on N^{εB29} modified insulins are given in
 15 Table 1.

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel} , was measured on a LiChrosorb RP18 (5μm, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10%
 20 acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time, t_0 , was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human} , was adjusted to at least $2t_0$ by varying the ratio between the A and
 25 B solutions. $k'_{rel} = (t_{derivative} - t_0) / (t_{human} - t_0)$.

The degree of prolongation of the blood glucose lowering effect was studied in rabbits. Each insulin derivative was tested by subcutaneous injection of 12 nmol thereof in each of six rabbits in the single day retardation test. Blood sampling for
 30 glucose analysis was performed before injection and at 1, 2, 4 and 6 hours after injection. The glucose values found are expressed as percent of initial values. The Index of

Protraction, which was calculated from the blood glucose values, is the scaled Index of Protraction (prolongation), see p. 211 in Markussen et al., Protein Engineering 1 (1987) 205-213. The formula has been scaled to render a value of 100 with 5 bovine ultralente insulin and a value of 0 with Actrapid® insulin (Novo Nordisk A/S, 2880 Bagsvaerd, Denmark).

The insulin derivatives listed in Table 1 were administered in solutions containing 3 Zn^{2+} per insulin hexamer, except those specifically indicated to be Zn-free.

10 For the very protracted analogues the rabbit model is inadequate because the decrease in blood glucose from initial is too small to estimate the index of protraction. The prolongation of such analogues is better characterized by the disappearance rate in pigs. $T_{50\%}$ is the time when 50% of the 15 A14 Tyr(^{125}I) analogue has disappeared from the site of injection as measured with an external γ -counter (Ribet, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P.J. Lefebre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes 20 Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

In Table 2 are given the $T_{50\%}$ values of a series of very protracted insulin analogues. The analogues were administered in solutions containing 3 Zn^{2+} per insulin hexamer.

Table 1

Insulin Derivative *)	Relative Lipophilicity	Blood glucose, % of initial				Index of protraction
		1h	2h	4h	6h	
N ^e B ²⁹ -benzoyl insulin	1.14					
N ^e B ²⁹ -phenylacetyl insulin (Zn-free)	1.28	55.4	58.9	88.8	90.1	10
N ^e B ²⁹ -cyclohexylacetyl insulin	1.90	53.1	49.6	66.9	81.1	28
N ^e B ²⁹ -cyclohexylpropionyl insulin	3.29	55.5	47.6	61.5	73.0	39
N ^e B ²⁹ -cyclohexylvaleroyl insulin	9.87	65.0	58.3	65.7	71.0	49
N ^e B ²⁹ -octanoyl insulin	3.97	57.1	54.8	69.0	78.9	33
N ^e B ²⁹ -decanoyl, des(B30) insulin	11.0	74.3	65.0	60.9	64.1	65
N ^e B ²⁹ -decanoyl insulin	12.3	73.3	59.4	64.9	68.0	60
N ^e B ²⁹ -undecanoyl, des(B30) insulin	19.7	88.1	80.0	72.1	72.1	80
N ^e B ²⁹ -lauroyl, des(B30) insulin	37.0	91.4	90.0	84.2	83.9	78
N ^e B ²⁹ -myristoyl insulin	113	98.5	92.0	83.9	84.5	97
N ^e B ²⁹ -chologoyl insulin	7.64	58.2	53.2	69.0	88.5	20
N ^e B ²⁹ -7-deoxycholoyl insulin (Zn-free)	24.4	76.5	65.2	77.4	87.4	35
N ^e B ²⁹ -lithocholoyl insulin (Zn-free)	51.6	98.3	92.3	100.5	93.4	115
N ^e B ²⁹ -4-benzoyl-phenylalanyl insulin	2.51	53.9	58.7	74.4	89.0	14
N ^e B ²⁹ -3,5-diiodotyrosyl insulin	1.07	53.9	48.3	60.8	82.1	27
N ^e B ²⁹ -L-thyroxy insulin	8.00					

Table 2

Derivative of Human Insulin	Relative hydrophobicity	Subcutaneous disappearance in pigs
5 600 μ M, 3Zn ²⁺ /hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	k'_{rel}	$T_{50\%}$, hours
10 N ^{εB29} decanoyl des(B30) insulin	11.0	5.6
N ^{εB29} undecanoyl des(B30) insulin	19.7	6.9
N ^{εB29} lauroyl des(B30) insulin	37	10.1
15 N ^{εB29} tridecanoyl des(B30) insulin	65	12.9
N ^{εB29} myristoyl des(B30) insulin	113	13.8
20 N ^{εB29} palmitoyl des(B30) insulin	346	12.4
N ^{εB29} succinimido- myristic acid insulin	10.5	13.6
25 N ^{εB29} myristoyl insulin	113	11.9
Human NPH		10

Solubility

The solubility of all the N^{εB29} modified insulins mentioned in Table 1, which contain 3 Zn²⁺ ions per insulin hexamer, exceeds 30 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation which further comprises 0.3% phenol as preservative, and 1.6% glycerol to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The ϵ -B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the ϵ -B29 amino group can also be an alkyl group.

5 Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means
10 of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

15 The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative
20 is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium
25 hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

30 Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterized in containing the Schizosaccharomyces pombe triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilization. A plasmid containing the POT-gene is available from a deposited E. coli strain (ATCC 39685). The plasmids furthermore contain the S. cerevisiae triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M. et al., Gene 73 (1988) 113-120) (see Fig. 1) except for the region defined by the ECoRI-XbaI restriction sites encompassing the coding region for signal/leader/product.

15 Synthetic DNA fragments were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron Letters 22 (1981) 1859-1869).

20 All other methods and materials used are common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989).

Analytical

25 Molecular masses of the insulins prepared were obtained by MS (mass spectroscopy), either by PDMS (plasma desorption mass spectrometry) using a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or by ESMS (electrospray mass spectrometry) using an API III Biomolecular Mass Analyzer (Perkin-Elmer Sciex
30 Instruments, Thornhill, Canada).

EXAMPLE 1

Synthesis of Ala^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA002 using the LaC212spx3 signal/leader.

5 The following oligonucleotides were synthesized:

- #98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCA
 CTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA
 AGAGGTTTCTTCTACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3})
 (SEQ ID NO:3)
- 10 #128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAA
 AGAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTC
 GTCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)
- #126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3})
 (SEQ ID NO:5)
- 15 #16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with
 20 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA).

- 2.5 µl of oligonucleotide #98 (2.5 pmol)
 2.5 µl of oligonucleotide #128 (2.5 pmol)
 10 µl of 10X PCR buffer
 16 µl of dNTP mix
 25 0.5 µl of Taq enzyme
 58.5 µl of water

One cycle was performed: 94°C for 45 sec., 49°C for 1 min, 72°C for 2 min.

Subsequently, 5µl of oligonucleotides #16 and #126 was added
 30 and 15 cycles were performed: 94°C for 45 sec., 45°C for 1 min, 72°C for 1.5 min. The PCR mixture was loaded onto a 2.5 %

agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μ l of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and Xba I according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp composed of a EcoRI/NcoI fragment encoding the synthetic yeast signal/leader gene LaC212spx3 (described in Example 3 of WO 89/02463) followed by a synthetic NcoI/XbaI fragment encoding the insulin precursor MI5, which has a SerAspAspAlaLys bridge connecting the B29 and the A1 amino acid residues (see SEQ ID NOS. 14, 15 and 16), inserted into the EcoRI/XbaI fragment of the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK188 is shown in Fig. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mixture was transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using standard DNA miniprep technique (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, Xba I, NcoI and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S. Biochemical Corp.) to contain the correct sequence for the Ala^{A21}, Asp^{B3} human insulin precursor and named pEA5.3.

The plasmid pKFN1627 is an E. coli - S. cerevisiae shuttle vector, identical to plasmid pKFN1003 described in EP patent No. 375718, except for a short DNA sequence upstream from the unique XbaI site. In pKFN1003, this sequence is a 178 bp
5 fragment encoding a synthetic aprotinin gene fused in-frame to the yeast mating factor alpha 1 signal-leader sequence. In pKFN1627, the corresponding 184 bp sequence encodes the insulin precursor MI5 (Glu^{B1}, Glu^{B28}) (i.e. B(1-29, Glu^{B1}, Glu^{B28})-SerAspAspAlaLys-A(1-21) fused in-frame to the mating factor
10 alpha 1 sequence (see SEQ ID NOS. 17, 18 and 19). The vector pKFN1627 is shown in Fig. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting DNA fragment of 412 bp was isolated. The yeast expression vector pKFN1627 was cut with the restriction
15 endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoI I/XbaI fragment and the
20 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mixture was transformed into E. coli as described above. Plasmid from the resulting E. coli was isolated using standard techniques, and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, Hpa I. The selected
25 plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, U.S. Biochemical) to contain the correct sequence for the Ala^{A21} Asp^{B3} human insulin precursor DNA and to be inserted after the DNA encoding the LaC212spx3 signal/leader DNA. The plasmid was named pEA5.3.2
30 and is shown in Fig. 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 20, 21 and 22. The plasmid pEA5.3.2 was transformed into S. cerevisiae strain MT663 as described in European patent

application having the publication No. 214826 and the resulting strain was named yEA002.

EXAMPLE 2

Synthesis of Ala^{A21} Thr^{B3} human insulin precursor from Yeast strain yEA005 using the Lac212spx3 signal/leader.

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTTCGTTACTCAACACTTGTGCGGTTCTCACTT
GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
10 CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)
#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAA
GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG
TCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)
#15 5'-GTCGCCATGGCTAAGAGATTTCGTTA-3' (Thr^{B3}) (SEQ ID
15 NO:8)
#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Ala^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The
20 DNA sequence encoding the Lac212spx3 signal/leader/Ala^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 23, 24 and 25. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the
25 resulting strain was named yEA005.

EXAMPLE 3

Synthesis of Gly^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA007 using the Lac212spx3 signal/leader.

30 The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTG
GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCT
ACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3}) (SEQ ID NO:3)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
5 AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID
NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

10 The DNA encoding Gly^{A21} Asp^{B3} human insulin precursor was
constructed in the same manner as described for the DNA
encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The
DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Asp^{B3}
human insulin precursor complex and the amino acid sequence
15 thereof are SEQ ID NOS. 26, 27 and 28. The plasmid pEA1.5.6 was
shown to contain the desired sequence, transformed into S.
cerevisiae strain MT663 as described in Example 1 and the
resulting strain was named yEA007.

EXAMPLE 4

20 Synthesis of Gly^{A21} Thr^{B3} human insulin precursor from Yeast
strain yEA006 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
25 GGTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)

30 #15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID
NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the Lac212spx3 signal/leader/Gly^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 29, 30 and 31. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA006.

10 EXAMPLE 5

Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) from Yeast strain yEA113 using the alpha factor leader.

15 A)

The following oligonucleotides were synthesized:

#220	5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3' (SEQ ID NO:10)
#263	5'-CACTTG GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTC
	TTCTACACTCCAAAGACTAGAGGTATCGTTGAA-3' (SEQ ID NO:11)
20 #307	5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT
	AGATTCGTTAACCAACAC-3' (SEQ ID NO:12)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Ave, Walk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlaid with 100 µl of mineral oil (Sigma Chemical Co, St. Louis, MO, USA). The plasmid pAK220 (which is identical to pAK188) consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader Lac212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor MI5 (see SEQ ID NOS. 14, 15 and 16) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA).

5 μ l of oligonucleotide #220 (100 pmol)
5 μ l of oligonucleotide #263 (100 pmol)
10 μ l of 10X PCR buffer
16 μ l of dNTP mix
5 0.5 μ l of Taq enzyme
0.5 μ l of pAK220 plasmid (identical to pAK188) as template (0.2
 μ g of DNA)
63 μ l of water

A total of 16 cycles were performed, each cycle comprising 1
10 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The
PCR mixture was then loaded onto a 2% agarose gel and subjected
to electrophoresis using standard techniques. The resulting DNA
fragment was cut out of the agarose gel and isolated using the
Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038,
15 USA) according to the manufacture's instructions. The purified
PCR DNA fragment was dissolved in 10 μ l of water and
restriction endonuclease buffer and cut with the restriction
endonucleases HindIII and XbaI according to standard
techniques. The HindIII/XbaI DNA fragment was purified using
20 The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp
comprising an EcoRI/HindIII fragment derived from pMT636
(described in WO 90/10075) encoding the yeast alpha factor
leader and part of the insulin precursor ligated to the
25 HindIII/XbaI fragment from pAK188 encoding the rest of the
insulin precursor MI5 (see SEQ ID NOS. 32, 33 and 34) inserted
into the vector cPOT. The vector pAK406 is shown in Fig. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp
encoding the synthetic yeast signal/leader Lac212sp_{x3}
30 (described in Example 3 of WO 89/02463) followed by the gene
for the insulin precursor B(1-29)-GluLysArg-A(1-21) (A21-Gly)
(see SEQ ID NOS. 35, 36 and 37) inserted into the vector cPOT.
The plasmid pAK233 is shown in Fig. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragments were ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA108 and is shown in Fig. 2. The DNA sequence encoding the alpha factor leader/Arg^{B31} single chain human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 38, 39 and 40. The plasmid pEA 108 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA)

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #307 (100 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
0.5 µl of Taq enzyme
0.2 µl of pEA108 plasmid as template (0.1 ug DNA)
63 µl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto an 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp composed of an EcoRI/NcoI fragment derived from pMT636 (described in WO 90/10075) (constructed by by introducing a NcoI site in the 3'-end of the alpha leader by site directed mutagenesis) encoding the alpha factor leader followed by a NcoI/XbaI fragment from pAK188 encoding the insulin precursor MI5 (see SEQ ID NOS. 41, 42 and 43) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK401 is shown in Fig. 3.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mixture was then transformed into a competent *E. coli* strain and plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in Fig. 3), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragments were ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA

ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA with the N-terminal extension GluGluAlaGluAlaGluAlaArg and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA113 and is shown in Fig. 3. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 44, 45 and 46. The plasmid pEA113 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA113.

EXAMPLE 6

Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) from Yeast strain yEA136 using the alpha factor leader.

The following oligonucleotide was synthesized:

25 #389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCGAAG
CTGAAAGATTTCGTTAACCAACAC-3' (SEQ ID NO:13)

The following PCR was performed using the Gene Amp PCR reagent kit

5 µl of oligonucleotide #220 (100 pmol)
30 5 µl of oligonucleotide #389 (100 pmol)
10 µl of 10X PCR buffer

16 μ l of dNTP mix
0.5 μ l of Taq enzyme
2 μ l of pEA113 plasmid as template (0.5 ug DNA)
63 μ l of water

5 A total of 12 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 37°C; and 2 minutes at 72°C.

The DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) was constructed in the same
10 manner as described for the DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain
15 human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) and the amino acid sequence thereof are SEQ ID NOS. 47, 48 and 49. The plasmid pEA136 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA136.

20 EXAMPLE 7

Synthesis of (A1,B1)-diBoc human insulin.

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The
25 reaction was conducted at room temperature and initiated by addition of 565 mg of di-~~tert~~-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 μ l of ethanolamine. The product was precipitated by addition of 1500 ml of acetone.
30 The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

(A1,B1)-diBoc insulin was purified by reversed phase HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μ m, pore size 100 Å) and equilibrated with elution buffer. The elution was performed using mixtures of ethanol and 1 mM aqueous HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of (A1,B1)-diBoc human insulin was obtained at a purity of 94.5%.

EXAMPLE 8

15 Synthesis of ($N^{\epsilon 829}$ -benzoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μ l of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 μ l DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

30 $N^{\epsilon 829}$ -benzoyl human insulin was purified by reversed phase HPLC as described in Example 7. A yield of 230 mg was obtained. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺

and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg.

Molecular mass, found by MS: 5911, theory: 5911.

5 EXAMPLE 9

Synthesis of (N^{6B29}-lithocholoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μ l of a mixture of N-
10 methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 μ l of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by
15 centrifugation and dried in vacuum. 331 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The
20 precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholoyl insulin was purified by reversed phase HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallization from 15% aqueous ethanol
25 containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

Molecular mass, found by MS: 6160, theory: 6166.

EXAMPLE 10

Synthesis of (N^{ε829}-decanoyl human insulin)₆, 3Zn²⁺.

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of
5 DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 18.0 mg of decanoic acid N-hydroxysuccinimide ester dissolved in 132 μl of DMF. The reaction was stopped after 60 minutes and the product
10 precipitated by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and
15 the product was then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by reversed phase HPLC as described in Example 7. A final yield of 254 mg of the title
20 product was obtained. The purity was 96.1%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn²⁺ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 217 mg.

25 Molecular mass, found by MS: 5962, theory: 5962.

EXAMPLE 11

Synthesis of des(B30) human insulin.

Synthesis of des(B30) human insulin was carried out as
30 described by Markussen (Methods in diabetes research, Vol. I,

Laboratory methods, part B, 404-410. Ed: J. Larner and S. Phol, John Wiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals were suspended in 25 ml of water and 1 M ammonia was added until a clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallization was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 μ m filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

EXAMPLE 12

Synthesis of (A1,B1)-diBoc des(B30) human insulin.

The title compound was synthesized by a method similar to that described in Example 7, using des(B30) porcine insulin as the starting material. The crude product was precipitated by acetone and dried in vacuum. The (A1,B1)-diBoc des(B30) human

insulin was purified by reversed phase HPLC as described in Example 7.

EXAMPLE 13

Synthesis of N^εB²⁹-decanoyl des(B30) human insulin.

5

400 mg of (A1,B1)-diBoc des(B30) human insulin was used as starting material for the synthesis of N^εB²⁹-decanoyl des(B30) human insulin, following the procedure described in Example 10. The crude product was precipitated by acetone, dried in vacuum
10 and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. N^εB²⁹-decanoyl des(B30) human insulin was then purified by reversed phase HPLC as described in Example 10.

Molecular mass, found by MS: 5856, theory: 5861.

15 **EXAMPLE 14**

Synthesis of N^εB²⁹-dodecanoyl des(B30) human insulin.

a. Immobilization of A. lyticus protease

13 mg of A. lyticus protease, dissolved in 5 ml of aqueous 0.2
20 M NaHCO₃ buffer, pH 9.4, was mixed with 4 ml of settled MiniLeak[®] Medium gel, which had been washed with the same buffer (MiniLeak is a divinylsulfone activated Sepharose CL 6B, obtained from KemEnTec, Copenhagen). The gel was kept in suspension by gentle stirring for 24 hours at room temperature.
25 Then, the gel was isolated by filtration, washed with water, and suspended in 20 ml of 1 M ethanolamine buffer, pH 9.4, and kept in suspension for 24 hours at room temperature. Finally, the gel was washed with water followed by 0.1 M acetic acid and stored at 4°C. The enzyme activity in the filtrate was 13% of

that in the initial solution, indicating a yield in the immobilization reaction of about 87%.

b. Immobilization of porcine trypsin

Porcine trypsin was immobilized to MiniLeak® Low to a degree of substitution of 1 mg per ml of gel, using the conditions described above for immobilization of A. lyticus.

c. Synthesis of Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) insulin using immobilized A. lyticus protease

To 200 mg of Glu(GluAla)₃Arg-B(1-29)-ThrArg-A(1-21) single-chain human insulin precursor, dissolved in 20 ml of 0.1 M NaHCO₃ buffer, pH 9.0, was added 4 ml of the gel carrying the immobilized A. lyticus protease. After the gel had been kept in suspension in the reaction mixture for 6 hours at room temperature the hydrolysis was complete, rendering Glu(GluAla)₃-Arg-B(1-29), ThrArg-A(1-21) human insulin (the reaction was followed by reversed phase HPLC). After the hydrolysis, the gel was removed by filtration. To the filtrate was added 5 ml of ethanol and 15 µL of 1 M ZnCl₂ and the pH was adjusted to 5.0 using HCl. The precipitation of the product was completed on standing overnight at 4°C with gentle stirring. The product was isolated by centrifugation. After one washing with 1 ml of ice cold 20% ethanol and drying in vacuo the yield was 190 mg.

d. Synthesis of N^{αA1}, N^{αB1}, N^{εB29}-tridodecanoyl Glu(GluAla)₃Arg-B(1-29), Thr-Arg-A(1-21) human insulin using dodecanoic acid N-hydroxysuccinimide ester

190 mg (30 µmol) of Glu(GluAla)₃Arg-B(1-29), ThrArg-A(1-21) insulin was dissolved in 1 ml of DMSO and 1.05 ml of a 0.572 M solution of N,N-diisopropylethylamine in DMF. The solution was cooled to 15°C and 36 mg (120 µmol) of dodecanoic acid N-hydroxysuccinimide ester dissolved in 0.6 ml of DMSO was added.

The reaction was completed within 24 hours. The lipophilic title compound was not isolated.

e. Synthesis of N^{εB29}-dodecanoyl des(B30) insulin

The product from the previous step, d., contained in approximately 2,65 ml of DMSO/DMF/N,N-diisopropylethylamine was diluted with 10.6 ml of a 50 mM glycine buffer comprising 20% ethanol and the pH adjusted to 10 with NaOH. After standing for 1 hour at room temperature 1 ml of MiniLeak gel, carrying 1 mg of immobilized trypsin per ml of gel, was added. The reaction mixture was stirred gently for 48 hours at room temperature. In order to isolate the desired product, the reaction mixture was applied to a reversed phase HPLC column (5 cm in diameter, 30 cm high), packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å). For the elution was used 20 mM Tris/HCl buffers, adjusted to pH 7.7 and comprising an increasing concentration of ethanol, from 40% to 44% (v/v), at a rate of 2000 ml/h. The major peak eluting at about 43-44% of ethanol contained the title compound. The fractions containing the major peak were pooled, water was added to reduce the ethanol concentration to 20% (v/v), and the pH was adjusted to 5.5. The solution was left overnight at -20°C, whereby the product precipitated. The precipitate was isolated by centrifugation at -8°C and dried in vacuo. The yield of the title compound was 90 mg.

Molecular mass, found by MS: 5892, theory: 5890.

EXAMPLE 15

Synthesis of N^{εB29}-(N-myristoyl-α-glutamyl) human insulin.

500 mg of (A1,B1)-diBoc human insulin was dissolved in 2.5 ml of DMSO and 428 μl of ethyl diisopropylamine, diluted with 2.5 ml of DMSO/DMF 1/1 (v/v), was added. The temperature was

adjusted to 15°C and 85 mg of N-myristoyl-Glu(OBut) N-hydroxysuccinimide ester, dissolved in 2.5 ml of DMSO/DMF 1/1 (v/v), was added. After 30 min the reaction mixture was poured into 60 ml of water, the pH adjusted to 5 and the precipitate isolated by centrifugation. The precipitate was dried in vacuo. The dried reaction mixture was dissolved in 25 ml of TFA, and the solution was left for 30 min at room temperature. The TFA was removed by evaporation in vacuo. The gelatinous residue was dissolved in 60 ml of water and the pH was adjusted to 11.2 using concentrated ammonia. The title compound was crystallized from this solution by adjustment of the pH to 8.5 using 6 N HCl. The product was isolated by centrifugation, washed once by 10 ml of water, and dried in vacuo. Yield 356 mg. Purity by HPLC 94%.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{12}\text{CONHCH}(\text{CH}_2\text{CH}_2\text{COOH})\text{CO}-$.

Molecular mass, found by MS: 6146, theory: 6148.

EXAMPLE 16

Synthesis of N^{B29}-undecanoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{B29}-dodecanoyl des(B30) human insulin as described in Example 14, by using undecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5876, theory: 5876.

EXAMPLE 17

Synthesis of N^{ε829}-tridecanoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{ε829}-
5 dodecanoyl des(B30) human insulin as described in Example 14,
by using tridecanoic acid N-hydroxysuccinimide ester instead of
dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5899, theory: 5904.

EXAMPLE 18

10 Synthesis of N^{ε829}-myristoyl des(B30) human insulin.

The title compound was synthesized analogously to N^{ε829}-
dodecanoyl des(B30) human insulin as described in Example 14,
by using myristic acid N-hydroxysuccinimide ester instead of
15 dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5923, theory: 5918.

EXAMPLE 19

Synthesis of N^{ε829}-palmitoyl des(B30) human insulin.

20 The title compound was synthesized analogously to N^{ε829}-
dodecanoyl des(B30) human insulin as described in Example 14,
by using palmitic acid N-hydroxysuccinimide ester instead of
dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5944, theory: 5946.

EXAMPLE 20

Synthesis of N^{εB29}-suberoyl-D-thyroxine human insulin.

a. Preparation of N-(succinimidylsuberoyl)-D-thyroxine.

5 Disuccinimidyl suberate (1.0 g, Pierce) was dissolved in DMF (50 ml), and D-thyroxine (2.0 g, Aldrich) was added with stirring at 20°C. The thyroxine slowly dissolved, and after 20 hours the solvent was removed by evaporation in vacuo. The oily residue was crystallized from 2-propanol to yield 0.6 g of N-
10 (succinimidylsuberoyl)-D-thyroxine, m.p. 128-133°C.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuberoyl)-D-thyroxine.

(A1,B1)-diBoc human insulin (200 mg) was dissolved in dry DMF (10 ml) by addition of triethylamine (20 μl) at room
15 temperature. Then, N-(succinimidylsuberoyl)-D-thyroxine (80 mg) was added. The reaction was monitored by reversed phase HPLC and when the reaction was about 90% complete, the solvent was removed in vacuo. To the evaporation residue, anhydrous trifluoroacetic acid (5 ml) was added, and the solution was
20 kept for 1 hour at room temperature. After removal of the trifluoroacetic acid in vacuo, the residue was dissolved in a mixture of 1M acetic acid (5 ml) and acetonitrile (1.5 ml), purified by preparative reversed phase HPLC and desalted on a PD-10 column. The yield of N^{εB29}-suberoyl-D-thyroxine human
25 insulin was 50 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: Thyrox-CO(CH₂)₆CO-, wherein Thyrox is thyroxine which is bound to the octanedioic acid moiety via an amide bond to
30 its α-amino group.

Molecular mass of the product found by MS: 6724, theory: 6723.

EXAMPLE 21

Synthesis of N⁶²⁹-(2-succinylamido)myristic acid human insulin.

a. Preparation of α -aminomyristic acid methyl ester, HCl.

5 To methanol (5 ml, Merck) at -10°C , thionyl chloride (0.2 ml, Aldrich) was added dropwise while stirring vigorously. Then, α -aminomyristic acid (0.7 g, prepared from the α -bromo acid by reaction with ammonia) was added. The reaction mixture was stirred at room temperature overnight, and then evaporated to
10 dryness. The crude product (0.7 g) was used directly in step b.

b. Preparation of N-succinoyl- α -aminomyristic acid methyl ester.

α -Aminomyristic acid methyl ester, HCl (0.7 g) was dissolved in chloroform (25 ml, Merck). Triethylamine (0.35 ml, Fluka) was
15 added, followed by succinic anhydride (0.3 g, Fluka). The reaction mixture was stirred at room temperature for 2 hours, concentrated to dryness, and the residue recrystallized from ethyl acetate/petroleum ether (1/1). Yield: 0.8 g.

c. Preparation of N-(succinimidylsuccinoyl)- α -aminomyristic
20 acid methyl ester.

N-succinoyl- α -aminomyristic acid methyl ester (0.8 g) was dissolved in dry DMF (10 ml, Merck, dried over 4Å molecular sieve). Dry pyridine (80 μl , Merck), and di(N-succinimidyl)carbonate (1.8 g, Fluka) were added, and the reaction
25 mixture was stirred overnight at room temperature. The evaporation residue was purified by flash chromatography on silica gel 60 (Merck), and recrystallized from 2-propanol/petroleum ether (1/1). Yield of N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester: 0.13
30 g, m.p. $64-66^{\circ}\text{C}$.

d. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester.

The reaction was carried out as in Example 20 b., but using N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester (16 mg) instead of N-(succinimidylsuberoyl)-D-thyroxine. After removal of the trifluoroacetic acid in vacuo, the evaporation residue was treated with 0.1M sodium hydroxide at 0°C to saponify the methyl ester. When the saponification was judged to be complete by reversed phase HPLC, the pH value in the solution was adjusted to 3, and the solution was lyophilized. After purification by preparative reversed phase HPLC and desalting on a PD-10 column, the yield of N^{εB29}-(2-succinylamido)myristic acid human insulin was 39 mg.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{11}\text{CH}(\text{COOH})\text{NHCOCH}_2\text{CH}_2\text{CO}-$.

Molecular mass of the product found by MS: 6130, theory: 6133.

EXAMPLE 22

Synthesis of N^{εB29}-octyloxycarbonyl human insulin.

20

The synthesis was carried out as in Example 20 b., but using n-octyloxycarbonyl N-hydroxysuccinimide (9 mg, prepared from n-octyl chloroformate (Aldrich) and N-hydroxysuccinimide), instead of N-(succinimidylsuberoyl)-D-thyroxine. The yield of N^{εB29}-octyloxycarbonyl human insulin was 86 mg.

The product of this example is thus human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_7\text{OCO}-$.

Molecular mass of the product found by MS: 5960, theory: 5964.

EXAMPLE 23

Synthesis of N^{ε829}-(2-succinylamido)palmitic acid human insulin.

a. Preparation of N-(succinimidylsuccinoyl)-α-amino palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c., using α-amino palmitic acid instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester.

10 The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester to give N^{ε829}-(2-succinylamido)palmitic acid human insulin.

15 The product of this example is thus human insulin wherein the ε-amino group of Lys⁸²⁹ has a substituent of the following structure: CH₃(CH₂)₁₃CH(COOH)NHCOCH₂CH₂CO-.

EXAMPLE 24

Synthesis of N^{ε829}-(2-succinylamidoethoxy)palmitic acid human
20 insulin.

a. Preparation of N-(succinimidylsuccinoyl)-2-aminoethoxy palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c. but
25 using 2-aminoethoxy palmitic acid (synthesized by the general procedure described by R. TenBrink, J. Org. Chem. 52 (1987) 418-422 instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-2-aminoethyloxypalmitic acid methyl ester.

The reaction was carried out as in Example 21 d., but using N-
5 (succinimidylsuccinoyl)-2-aminoethyloxypalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)- α -aminomyristic acid methyl ester to give N^{εB29}-(2-succinylamidoethoxy)palmitic acid human insulin.

The product of this example is thus human insulin wherein the
10 ϵ -amino group of Lys^{B29} has a substituent of the following structure: $\text{CH}_3(\text{CH}_2)_{13}\text{CH}(\text{COOH})\text{NHCH}_2\text{CH}_2\text{OCOCH}_2\text{CH}_2\text{CO}-$.

EXAMPLE 25

Synthesis of N^{εB29}-lithocholoyl- α -glutamyl des(B30) human insulin.

15

The synthesis was carried out as in Example 13 using N-lithocholoyl-L-glutamic acid α -N-hydroxysuccinimide ester, γ -tert-butyl ester instead of decanoic acid N-hydroxysuccinimide ester.

20 The product of this example is thus des(B30) human insulin wherein the ϵ -amino group of Lys^{B29} has a substituent of the following structure: lithocholoyl-NHCH(CH₂CH₂COOH)CO-.

Molecular mass of the product found by MS: 6194, theory: 6193.

EXAMPLE 26

Synthesis of N^{ε829}-3,3',5,5'-tetraiodothyroacetyl human insulin.

The synthesis was carried out as in Example 10 using 3,3',5,5'-
5 tetraiodothyroacetic acid N-hydroxysuccinimide ester, instead
of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6536, theory: 6538.

EXAMPLE 27

Synthesis of N^{ε829}-L-thyroxyl human insulin.

10

The synthesis was carried out as in Example 10 using Boc-L-
thyroxine N-hydroxysuccinimide ester, instead of decanoic acid
N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6572, theory: 6567.

15 **EXAMPLE 28**

A pharmaceutical composition comprising 600 nmol/ml of N^{ε829}-
decanoyl des(B30) human insulin, 1/3Zn²⁺ in solution.

N^{ε829}-decanoyl des(B30) human insulin (1.2 μmol) was dissolved in
20 water (0.8 ml) and the pH value was adjusted to 7.5 by addition
of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μl) and a
solution containing 0.75% of phenol and 4% of glycerol (0.8 ml)
was added. The pH value of the solution was adjusted to 7.5
using 0.2 M sodium hydroxide and the volume of the solution was
25 adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and
transferred aseptically to a cartridge or a vial.

EXAMPLE 29

A pharmaceutical composition comprising 600 nmol/ml of N^εB²⁹-decanoyl human insulin, $\frac{1}{2}$ Zn²⁺ in solution.

5 1.2 μ mol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. A solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide
10 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

EXAMPLE 30

A pharmaceutical composition comprising 600 nmol/ml of N^εB²⁹-
15 lithocholoyl human insulin in solution.

1.2 μ mol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added
20 0.8 ml of a stock solution containing 0.75 % cresol and 4% glycerol in water. Finally, the pH value was again adjusted to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and
25 transferred aseptically to a cartridge or a vial.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(ii) TITLE OF INVENTION: ACYLATED INSULIN

(iii) NUMBER OF SEQUENCES: 49

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBERS: DK 1044/93 and US 08/190,829
- (B) FILING DATES: 09-SEP-1993 and 02-FEB-1994

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Jørgensen, Dan et al.
- (C) REFERENCE/DOCKET NUMBER: 3985.204-WO,DJ

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu
1				5					10					15	
Glu Asn Tyr Cys Xaa															
20															

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa	Val	Xaa	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr
1				5					10					15	
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Xaa															
20 25 30															

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 110 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCTAAGAG	ATTCGTTGAC	CAACACTTGT	GCGGTTCTCA	CTTGTTGAA	GCTTTGTACT	60
TGGTTTGTGG	TGAAAGAGGT	TTCTTCTACA	CTCAAAGTC	TGACGACGCT		110

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGCGGGCTG CGTCTAAGCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60
AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGCCATGG CTAAGAGATT CGTTG 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCTCTAGA GCCTGCGGGC TCGTCT 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 110 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCTAAGAG ATTCGTTACT CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60
TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 100

60

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGCCATGG CTAAGAGATT CGTTA

25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGGGCTG CGTCTAACCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC

60

AACATTGTTT AACGATACCC TTAGCGTCGT CAGACTTTGG

100

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGTACGTTT TAGAGCCTGC GGGCTGC

27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTGGTTG AAGCTTTGTA CTTGGTTTGT GGTGAAAGAG GTTCTTCTA CACTCCAAAG 60
ACTAGAGGTA TCGTTGAA 78

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCTG AAGCTAGATT CGTTAACCAA 60
CAC 63

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCGA AGCTGAAAGA TTCGTTAACC 60
AACAC 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

62

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC 60
 AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC 112
 Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
 1 5 10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG 160
 Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
 15 20 25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC 208
 Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
 30 35 40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC 256
 Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
 45 50 55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC 304
 Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
 60 65 70 75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT 352
 Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
 80 85 90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT AAC TAGACGCAGC 401
 Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 95 100

CCGCAGGCTC TAGA 415

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1 5 10 15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45

Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60

63

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Asn
 100

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACATT GATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC	60
--	----

64

AATATAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	112
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA GAA GTT AAC CAA CAC TTG	352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu	
80 85 90	
TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA	400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT GAA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA	448
Gly Phe Phe Tyr Thr Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu	
110 115 120	
CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT	496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys	
125 130 135	
AAC TAGACGCAGC CCGCAGGCTC TAGA	523
Asn	
140	

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1 5 10 15

65

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
 20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
 35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80

Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu Cys Gly Ser His Leu
 85 90 95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
 100 105 110

Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
 115 120 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 130 135 140

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG	120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG	180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCTA AAGCTACAAC GACAAAACGG	240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTGTAACG	300
ACGATTTCTT CTTCCCATTA GAAACCTATT CTCTCTTCAA TTGGTTGTGA ACACGCCAAG	360
AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGACTTTT	420
CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT	480
TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT	523

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                  1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
                  15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
                  30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
                  45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
                  60             65             70             75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                  80             85             90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
                  95             100
CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

67

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1           5           10           15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
          20           25           30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
          35           40           45
Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
          50           55           60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
          65           70           75           80
Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
          85           90           95
Tyr Gln Leu Glu Asn Tyr Cys Ala
          100

```

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCA GTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA      300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC      360
AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT          415

```

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:

68

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATATCAA TTTCATACAC      60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                  1             5             10
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
              15             20             25
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
              30             35             40
GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
              45             50             55
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
              60             65             70             75
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
              80             85             90
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
              95             100
CCGCAGGCTC TAGA      415

```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

69

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1 5 10 15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Ala
 100

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

70

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT	352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr	
80 85 90	
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC	401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly	
95 100	
CCGCAGGCTC TAGA	415

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 104 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
1 5 10 15

71

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 20 25 30
 Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45
 Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60
 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Gly
 100

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 415 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAATC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 415 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT	352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr	
80 85 90	
TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC	401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly	
95 100	
CCGCAGGCTC TAGA	415

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 104 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala	
1 5 10 15	
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser	
20 25 30	

73

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
 35 40 45
 Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
 50 55 60
 Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
 65 70 75 80
 Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
 85 90 95
 Tyr Gln Leu Glu Asn Tyr Cys Gly
 100

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 415 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TAGCTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC	120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA	180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT	240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA	300
GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC	360
AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT	415

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 523 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS

(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAAA TTTCATACAC	60
AATATAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	112
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA TTC GTT AAC CAA CAC TTG	352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu	
80 85 90	
TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA	400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA	448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu	
110 115 120	
CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT	496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys	
125 130 135	
AAC TAGACGCAGC CCGCAGGCTC TAGA	523
Asn	
140	

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 140 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

75

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1 5 10 15
 Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
 20 25 30
 Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
 35 40 45
 Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 50 55 60
 Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80
 Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
 85 90 95
 Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
 100 105 110
 Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
 115 120 125
 Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 130 135 140

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG	60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG	120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTA CTTTGCC GTGTTTAAGG	180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG	240
TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTGTAACG	300
ACGATTTCTT CTTCCCCATA GAAACCTATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG	360
AGTGAACCAA CTTGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGAGGTTT	420
CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT	480

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT

523

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 409 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..385

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC	60
AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC	256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His	
45 50 55	
TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC	304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr	
60 65 70 75	
ACT CCT AAG GAA AAG AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC	352
Thr Pro Lys Glu Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile	
80 85 90	
TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC CCGCAGGCTC	405
Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly	
95 100	
TAGA	409

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 amino acids

77

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1             5             10             15
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
      20             25             30
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
      35             40             45
Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
      50             55             60
Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Glu Lys
      65             70             75             80
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
      85             90             95
Leu Glu Asn Tyr Cys Gly
      100

```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC      120
GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACCTC TAAGGCCTTC TCAGAGACTA      180
GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT      240
GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA      300
GATGTGAGGA TTCCTTTTCT CTCCATAGCA ACTTGTTACA ACATGAAGAT AGACAAGAAA      360
CATGGTTAAC CTTTTGATGA CACCAATCTG CGTCGGGCGT CCGAGATCT      409

```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 511 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      109
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
              1              5              10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
              15              20              25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
              30              35              40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
              45              50              55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
              60              65              70              75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG      349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
              80              85              90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      397
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
              95              100              105

GGT TTC TTC TAC ACT CCA AAG ACT AGA GGT ATC GTT GAA CAA TGT TGT      445
Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys
              110              115              120

ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGC AAC      487
Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
              125              130              135

TAGACGCAGC CCGCAGGCTC TAGA      511

```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 137 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1           5           10           15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
      20           25           30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
      35           40           45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
      50           55           60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
      65           70           75           80
Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
      85           90           95
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
      100          105          110
Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser
      115          120          125
Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
      130          135

```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 511 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```

CTTAAGGTAA GTTCTTATCA AGTTTGTTC TCTAATGTTT GATAGTTAAA GTATGTGTTA      60
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTA AAAATGA CGTCAA AATA AGCGTCGTAG      120

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80

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTAAAGGCCG 180
 ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA 240
 AAGGTTGTCG TGTATTATGC CCAATAACAA ATATTATGA TGATAACGGT CGTAACGACG 300
 ATTTCTTCTT CCCCATAGGT ACCGATTCTC TAAGCAATTG GTTGTGAACA CGCCAAGGGT 360
 GAACCAACTT CGAAACATGA ACCAAACACC ACTTTCTCCA AAGAAGATGT GAGGTTTCTG 420
 ATCTCCATAG CAACTTGTTA CAACATGAAG ATAGACAAGA AACATGGTTA ACCTTTTGAT 480
 GACGTTGATC TCGTCGGGGC GTCCGAGATC T 511

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACATCAA TTTCATACAC 60
 AATATAAACG ATTAAGAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA 112
 Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
 1 5 10
 TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA 160
 Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
 15 20 25
 GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT 208
 Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
 30 35 40
 TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA 256
 Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
 45 50 55
 AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT 304
 Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60 65 70 75
 AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG 352
 Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
 80 85 90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC GGT GAA AGA	400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg	
95 100 105	
GGT TTC TTC TAC ACT CCT AAG TCT GAC GAT GCT AAG GGT ATT GTC GAG	448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu	
110 115 120	
CAA TGC TGT ACC TCC ATC TGC TCC TTG TAC CAA TTG GAA AAC TAC TGC	496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys	
125 130 135	
AAC TAGACGCAGC CCGCAGGCTC TAGA	523
Asn	
140	

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser	
1 5 10 15	
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln	
20 25 30	
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe	
35 40 45	
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu	
50 55 60	
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val	
65 70 75 80	
Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu	
85 90 95	
Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr	
100 105 110	
Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser	
115 120 125	
Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	
130 135 140	

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60
TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAATAAAGCGTCG      120
TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG      180
CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG      240
TAAAAGGTTG TCGTGTTTAT TGCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG      300
ACGATTTCTT CTTCCCCATA GGTACCGATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG      360
GGTGAACCAA CTTGAAACA TGAACCAAAC GCCACTTTCT CCAAAGAAGA TGTGAGGATT      420
CAGACTGCTA CGATTCCCAT AACAGCTCGT TACGACATGG AGGTAGACGA GGAACATGGT      480
TAACCTTTTG ATGACGTTGA TCTGCGTCGG GCGTCCGAGA TCT                          523

```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 535 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..511

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```

GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT      60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      109
          Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
          1              5              10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
          15              20              25

```


83

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp 30 35 40	205
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr 45 50 55	253
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala 60 65 70 75	301
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu 80 85 90	349
GCT AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT Ala Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala 95 100 105	397
TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG ACT Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr 110 115 120	445
AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln 125 130 135	493
TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA Leu Glu Asn Tyr Cys Asn 140 145	535

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser 1 5 10 15	
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln 20 25 30	
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe 35 40 45	
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu 50 55 60	

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65 70 75 80
 Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Arg Phe Val Asn
 85 90 95
 Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
 100 105 110
 Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu
 115 120 125
 Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
 130 135 140
 Asn
 145

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 535 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CTTAAGGTAA GTTCTTATCA AGTTTGTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA	60
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAATA AGCGTCGTAG	120
GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTAAAGGCCG	180
ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA	240
AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG	300
ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTGCACTTC GATCTAAGCA	360
ATTGTTGTG AACACGCCAA GGGTGAACCA ACTTCGAAAC ATGAACCAA CACCACTTTC	420
TCCAAAGAAG ATGTGAGGTT TCTGATCTCC ATAGCAACTT GTTACAACAT GAAGATAGAC	480
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(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 538 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 77..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT	60
ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA	109
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu	
1 5 10	
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA	157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu	
15 20 25	
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT	205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp	
30 35 40	
TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA	253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr	
45 50 55	
AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT	301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala	
60 65 70 75	
AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA	349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu	
80 85 90	
GCT GAA AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA	397
Ala Glu Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu	
95 100 105	
GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG	445
Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys	
110 115 120	
ACT AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC	493
Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr	
125 130 135	
CAA TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA	538
Gln Leu Glu Asn Tyr Cys Asn	
140 145	

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 amino acids

86

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

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Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1             5             10             15
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
          20             25             30
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
          35             40             45
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
          50             55             60
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
          65             70             75             80
Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Glu Arg Phe Val
          85             90             95
Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
          100            105            110
Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val
          115            120            125
Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr
          130            135            140
Cys Asn
          145

```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 538 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

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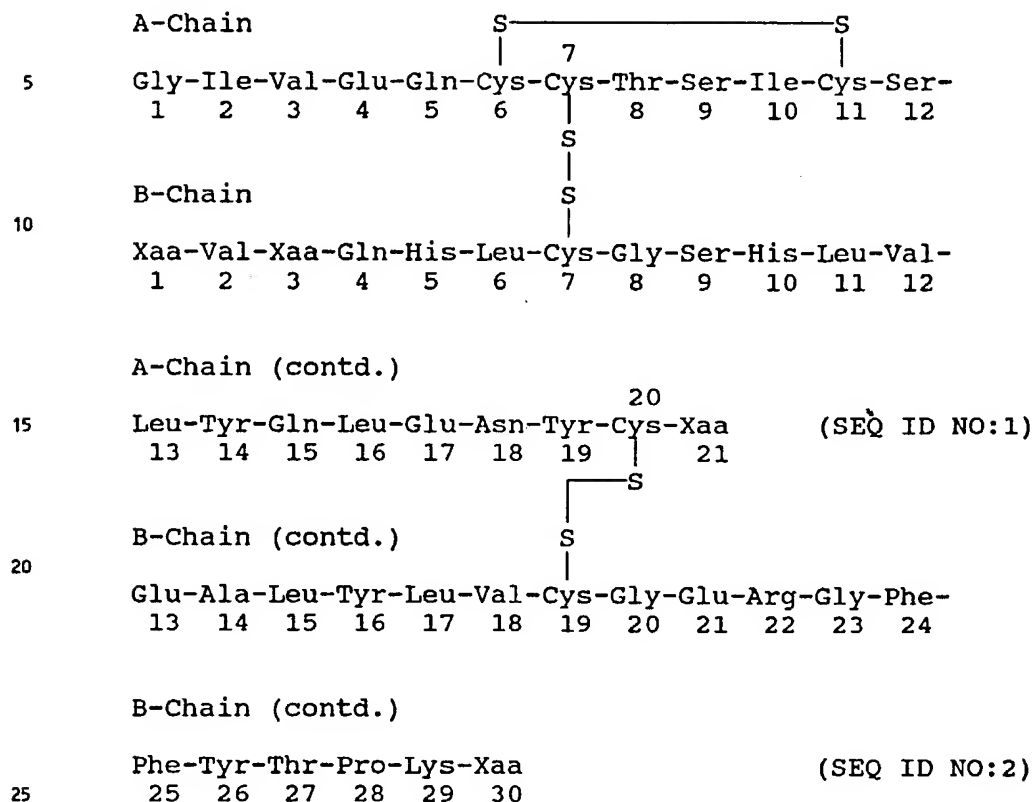
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TATTTGCTAA TTTTCTTACT CTAAGGAAG TAAAAATGA CGTCAAAATA AGCGTCGTAG      120
GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG      180

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ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA	240
AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG	300
ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTGACTTC GACTTTCTAA	360
GCAATTGGTT GTGAACACGC CAAGGGTGAA CCAACTTCGA AACATGAACC AAACACCACT	420
TTCTCCAAAG AAGATGTGAG GTTTCTGATC TCCATAGCAA CTTGTTACAA CATGAAGATA	480
GACAAGAAAC ATGGTTAACC TTTTGATGAC GTTGATCTGC GTCGGGCGTC CGAGATCT	538

CLAIMS

1. An insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

30 Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ -amino group of Lys^{B29}, (b) any amino acid residue
 35 which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ -amino group of Lys^{B29} has a lipophilic substituent ; and
 any Zn²⁺ complexes thereof,

provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn^{2+} complex.

2. The insulin derivative according to claim 1, wherein

5 Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is a non-codable, lipophilic
10 amino acid having from 10 to 24 carbon atoms and an acyl group is bound to the ϵ -amino group of Lys^{B29}, wherein the acyl group is an acyl group of a monocarboxylic acid with up to 4 carbon atoms or of a dicarboxylic acid with up to 5 carbon atoms.

3. The insulin derivative according to claim 1, wherein

15 Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is deleted or is any amino acid
20 residue which can be coded for by the genetic code except Lys, Arg and Cys and the ϵ -amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

4. The insulin derivative according to claim 2, wherein Xaa at position B30 is selected from the group consisting of α -amino
25 decanoic acid, α -amino dodecanoic acid, α -amino tetradecanoic acid and α -amino hexadecanoic acid.

5. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ -amino group of Lys^{B29} is selected from the group consisting of formyl, acetyl, propionyl and n-
30 butyryl.

6. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ -amino group of Lys^{B29} is an acyl group of succinic acid.
7. The insulin derivative according to claim 3, wherein Xaa at position B30 is deleted.
8. The insulin derivative according to claim 3, wherein Xaa at position B30 is Asp, Glu, or Thr.
9. The insulin derivative according to claim 3, wherein the lipophilic substituent bound to the ϵ -amino group of Lys^{B29} is an acyl group derived from a carboxylic acid having at least 6 carbon atoms.
10. The insulin derivative according to claim 9, wherein the acyl group, which may be branched, comprises a main chain of carbon atoms 8 - 24 atoms long.
11. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a fatty acid having at least 6 carbon atoms.
12. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.
13. The insulin derivative according to claim 9, wherein the acyl group is selected from the group comprising dodecanoic acid, tridecanoic acid and tetradecanoic acid.
14. The insulin derivative according to claim 1, wherein Xaa at position A21 is Ala, Gln, Gly or Ser.
15. The insulin derivative according to claim 1, wherein Xaa at position B3 is Asp, Gln or Thr.

16. The insulin derivative according to claim 1, wherein Xaa at position B1 is deleted.

17. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a
5 therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

18. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a
10 therapeutically effective amount of an insulin derivative according to claim 1, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

19. A method of treating diabetes in a patient in need of such
15 a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

20. A method of treating diabetes in a patient in need of such
20 a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

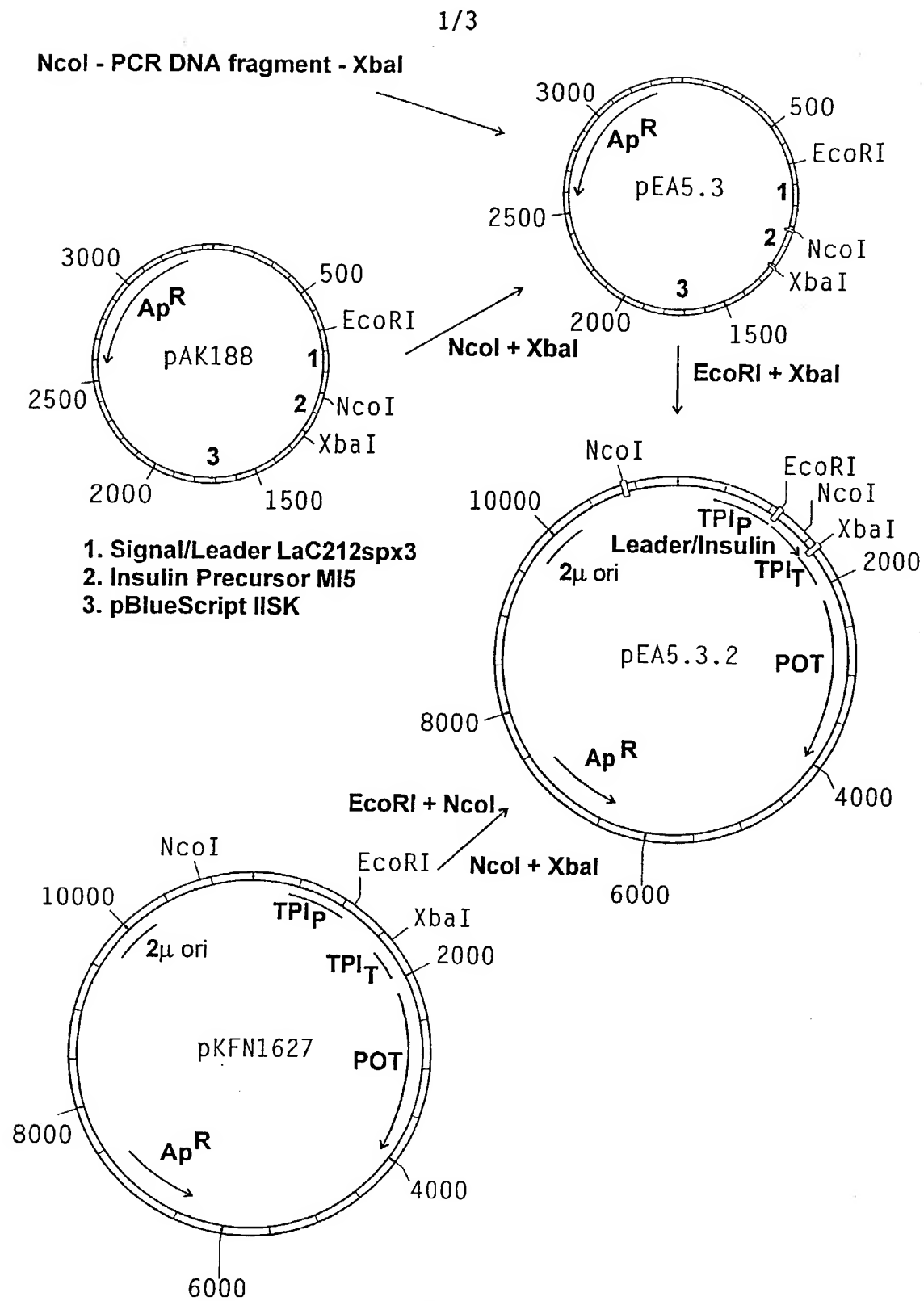


Fig. 1

2/3

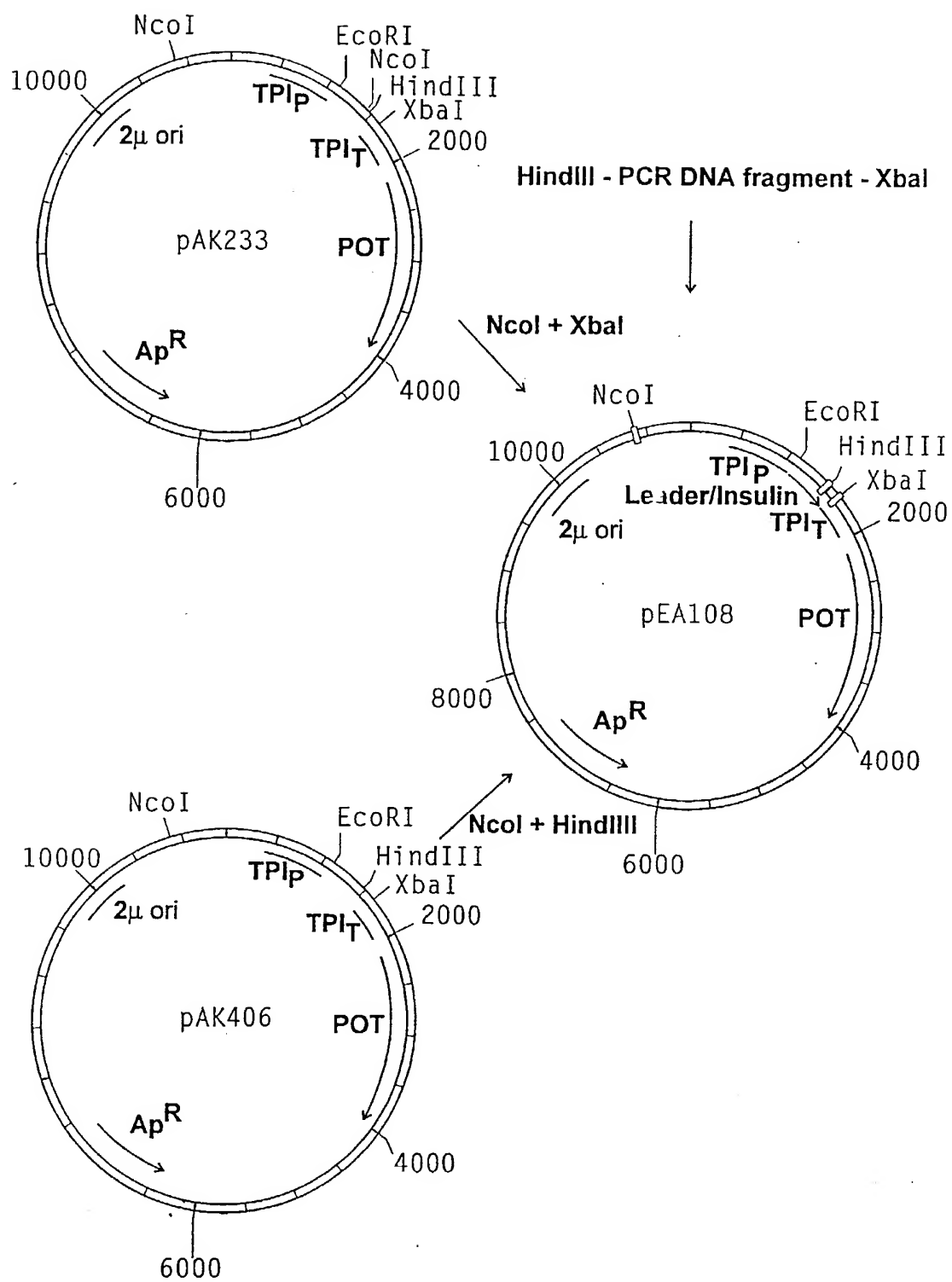


Fig. 2

3/3

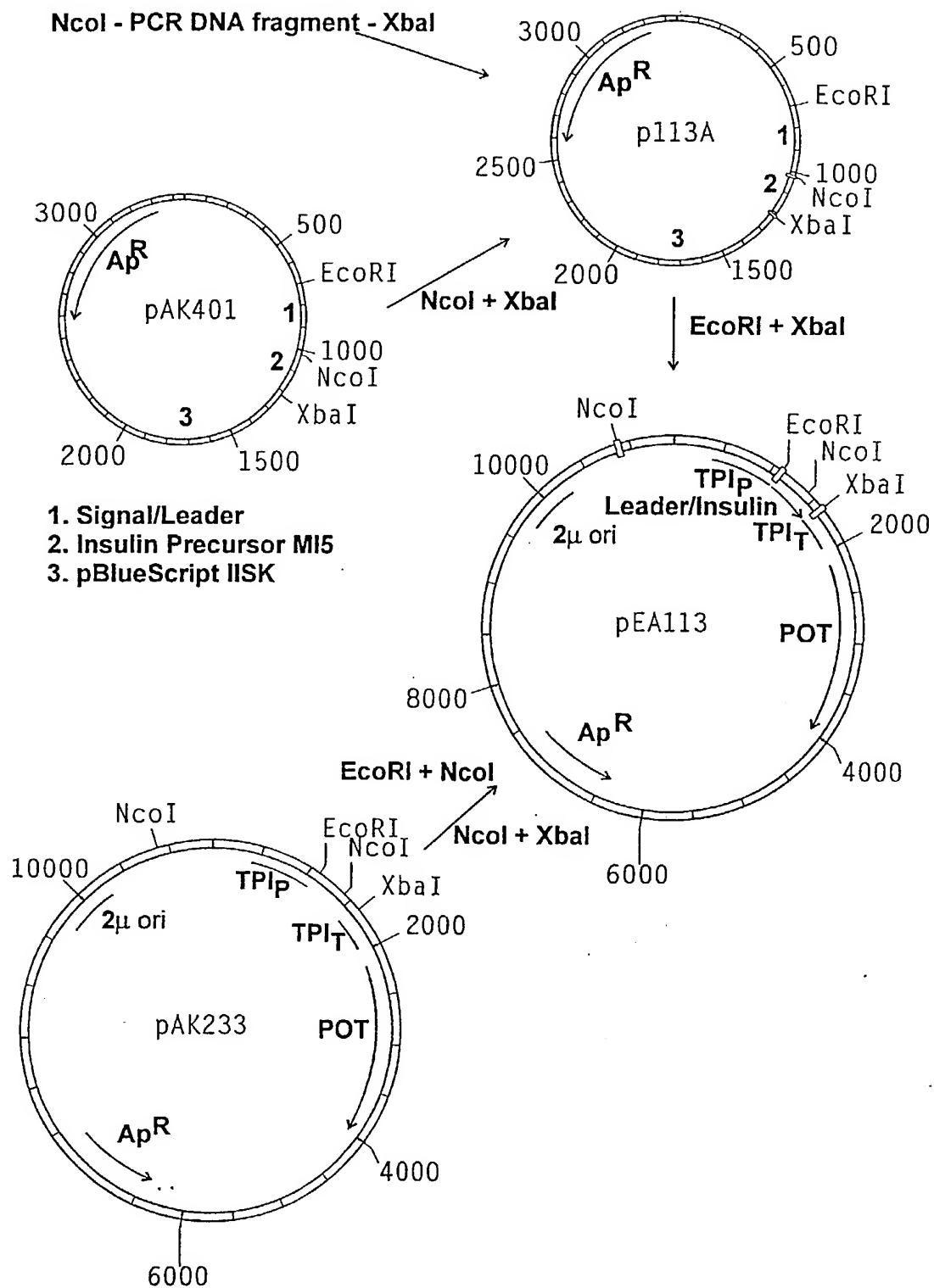


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, A61K 38/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, CA, CLAIMS, JAPIO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Patent Abstracts of Japan, Vol 14, No 7, C-673, abstract of JP, A, 1--254699 (KODAMA K.K.), 11 October 1989 (11.10.89) --	1-18
A	US, A, 3823125 (N. H. GRANT ET AL), 9 July 1974 (09.07.74) --	1-18
A	DE, B2, 2209835 (BAYER AG), 29 April 1976 (29.04.76) --	1-18
A	US, A, 3868356 (D. G. SMYTH), 25 February 1975 (25.02.75) --	1-18

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 December 1994

Date of mailing of the international search report

05-01-1995

Name and mailing address of the ISA/
Swedish Patent Office
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Authorized officer

Elisabeth Carlborg
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0127535 (HADASSAH MEDICAL ORGANIZATION), 5 December 1984 (05.12.84) ----- -----	1-18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00347

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 19, 20
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/11/94

International application No.

PCT/DK 94/00347

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US-A-	3823125	09/07/74	NONE	
DE-B2-	2209835	29/04/76	AT-B- 333987	27/12/76
			BE-A- 795997	27/08/73
			CH-A- 579916	30/09/76
			FR-A,B- 2181778	07/12/73
			GB-A- 1374385	20/11/74
			JP-A- 48097889	13/12/73
			NL-A- 7302898	04/09/73
			SE-B,C- 421690	25/01/82
			US-A- 3907763	23/09/75
US-A-	3868356	25/02/75	AT-B- 339512	25/10/77
			AU-B- 472582	27/05/76
			AU-A- 3821372	26/07/73
			BE-A- 778538	26/07/72
			CH-A- 547777	11/04/74
			DE-A- 2204053	17/08/72
			FR-A,B- 2123524	08/09/72
			GB-A- 1381274	22/01/75
			NL-A- 7201179	01/08/72
			SE-B,C- 382452	02/02/76
EP-A2-	0127535	05/12/84	SE-T3- 0127535	
			CA-A- 1223200	23/06/87
			JP-B- 6078238	05/10/94
			JP-A- 60069028	19/04/85
			US-A- 4579730	01/04/86